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# Phytochemical and Antimicrobial Activity of *Medicago sativa* (Alfalfa) as Source of Animal Food Against Some Animal Pathogens

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**Abstract:** Many plants have so far been used for the treatment and management of various ailments since the beginning of human civilization. One of the common problems in the medical world, spreading of bacterial resistance against antibiotics, so one of the most important steps in microbiological researches is to find a new antimicrobial compound with minimal side effects. Due to the presence of biological active compounds in plant *Medicago sativa* and its use in traditional objective medicine and animal nutrition, it seems that this plant contain considerable antimicrobial capacity. So the aim of this study is to investigate the antimicrobial activity of *Medicago sativa* aqueous and solvent extracts on some medically important animal pathogens and to determine some phytochemical compounds. Hot and cold aqueous extracts in addition to successive solvent extracts.( acetone, ether, ethanol and chloroform)of *Medicago sativa* were evaluated for their antimicrobial activity against some medically important pathogens isolated from animals and poultry farms (*Staphylococcus aureus, Escherichia coli, Streptococcus pyogens, Pseudomonas aerogenes, Salmonella typhimurium, Enterococcus, Bacillus cereus, Klebsiella pneumonia and Candida albicans)*by agar well diffusion method. The hot aqueous extract is the most effective against *Staphylococcus aureus* followed by cold aqueous extract showed significant antibacterial activity against *B.cereus, S.typhimurium, K.pneumoniae, P.aerogenes and E.coli* followed by ethanol extract followed by acetone extract followed by chloroform while the ether extract did not show any significant antibacterial or antifungal activity against all examined microbes except *Enterococcus*. However ethanol extract is the only extract showed high antifungal effect against *Candida albicans***.** Also, phytochemical compound of ethanol extract was determined, results of the chemical tests explain the *M. sativa* extracts contain flavonoids, tannins, alkaloids, saponin and glycosides compounds. So it could be concluded that the *M. sativa* extract possess remarkable antimicrobial activity against microbial pathogens and to be introduced as an alternative to chemical antimicrobial drugs, is required wider investigation.

**Key words:** Natural Antimicrobial · Father Of Food · New Antibiotic · Alfalfa

## INTRODUCTION

Microbes are the most common cause of infectious diseases which participate in about half percent of the deaths cases in animals. As well as morbidity and mortality due to diarrhea in many developing countries which act as a major problem, The infections due to variety of bacterial etiologic agents such as pathogenic *Escherichia coli(E. coli),Salmonella* spp. *and Staphylococcus aureus (S. aureus)* are most common [1]. Also systemic fungal infections due to *Candida albicans* (*C. albicans)* have emerged as important causes of morbidity and mortality [2].

B. cereus was identified as an in frequent abortigenic agent in cattle. Necrotizing placentitis with no or sporadic lesions in fetal tissues [3].

K. pneumoniae is a common cause of clinical mastitis in dairy cattle. Wood products are considered to be the main source of Klebsiella on dairy farms [4].

Antibiotic resistance has become a global concern. as multi-drug resistant pathogen mostly affects the clinical efficacy of many existing antibiotics [5]. Throughout the history of mankind many infectious diseases have been known to be treated with herbal remedies where medicinal plants still act as the most common source of antimicrobial agents. This traditional

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health remedies use is the most popular for 80% of world population all over the world and was reported to have minimal side effects pharmacological or biological tests have been submitted to approximately 20% of the plants found in the world. A continuous effort is done by the microbiologists using the systemic screening of antimicrobial plant extracts to find new compounds have the potential to act against multidrug resistant pathogenic bacteria and fungi [6].

*M. sativa* can survive more than 20 years, depending on climate and variety so it was described as a perennial forage legume with height up to 1 m (3 ft) and deeply root. These make it very resistant, especially to droughts. Also it is called as "Father of all food" [7].

*M. sativa* (Leguminosae), one of the most medicinal plant traditionally used to cure kidney pain, improve the memory, cough, sore muscles, as anti-inflammatory, antidiabetic, rejuvenator, antioxidant,, antimicrobial and in CNS disorders. In addition to its long tradition use as ayurvedic and homoeopathic medicine in CNS disorders [8].

*M. sativa* was reported phytochemicaly as it contain tannin, flavonoids, digestive enzymes, alkaloids, coumarins, phytosterols, phytoestrogens, triterpenes and saponins. The reduction of cholesterol absorption and atherosclerotic plaque formation in the arteries due to the ingestion of *M. sativa* was reported by several clinical and animal studies. More over its benefits in convalescence, cardiovascular complaints, diabetes and debility and also used as a tonic during anemia and after blood loss [8]. Tannins have been reported to be bacteriostatic or bactericidal against S. aureus [9]. The antimicrobial mechanisms of tannins can be summarized as follows. (i) The astringent property of the tannin may induce complexation with enzymes or substrates. Many microbial enzymes in raw culture filtrates or in purified forms are inhibited when mixed with tannins. (ii) A tannin's toxicity may be related to its action on the membranes of the microorganisms. (iii) Complexation of metal ions by tannins may account for tannin toxicity [10].

*Medicago* sativa was reported to have selective toxicity in dog cancer cells grown *in vitro* and anti-tumor activity against certain types of leukemia cells in mice [8].

So the aim of this study is to investigate the antimicrobial activity of *Medicago sativa* aqueous and solvent extracts on some medically important animal pathogens(*S. aureus, E coli, S. pyogens, P.aerogenes, Salmonella typhimurium, Enterococcus, B.cereus, K. pneumoniae and C. albicans)*by agar well diffusion method.

## MATERIALS AND METHODS

**Collection of Plant Materials:** Fresh plant materials were collected from farm in Giza. The collected different parts of plant were washed with distilled water, air dried, grinded to a fine powder using pestle and mortar and stored in air-tight bottles at room temperature.

**Aqueous Extraction:** About 10 ml of each hot and cold distilled water was added to 5g of the selected plant in sterile test tubes. The tubes were kept for 1 week at room temperature until use.

**Solvent Extraction:** About 5 g of dried plant material was extracted with 10 ml of each solvent (Ether, Acetone, Chloroform and Ethanol) kept for 24 h. Then, it was filtered using Watman No.1 filter paper. The solvent was evaporated to make the final volume as 1/2 of the original volume [11].

**Preparation of Inoculums:** The inoculums (bacterial strains and fungi) were isolated from large animals and poultry farms on the outskirts of Cairo. The strains of bacteria (*S. aureus*, *E. coli*, *S. pyogens, P.aerogenes, Salmonella typhimurium, Enterococcus, B. cereus, K. pneumoniae)*& fungi (*C. albicans)* were inoculated on Sabaroud dextrose agar (SAB) (Purchased from Watin – Biolife Company produced by Jalil Medicals Company)and in nutrient broth (Purchased from Watin – Biolife Company produced by Jalil Medicals Company).for overnight at 37°C for bacteria and 25°C for fungi.

**Antimicrobial Screening:** The preliminary study of antimicrobial activity of different extracts of *M. sativa* was performed by using agar well diffusion method [12]. The sensitivity of all extracts was tested against (*S. aureus*, *E. coli*, *S. pyogens, P.aerogenes, Salmonella typhimurium, Enterococcus, B. cereus, K. pneumonia )*& fungi (C. *albicans).* The anti-microbial activity was measured by the inhibition zones produced in milliliter. All experiments were duplicated. Ciprofloxacin (10 µ g) and penicillin (10 µ g) used as positive control while distilled water (100 µg) used as negative control for antibacterial screening aqueous extract while (0.01%) DMSO used as negative control in solvent extracts. Nystatin (10 µg) was used as positive control while distilled water (100 µg) used as negative control for antifungal screening. All chemicals used (Purchased from Witan – Biolife Company produced by Jalil Medicals Company).

**Phytochemical Tests**

**Tannins Test:**A modified methods stated in [13] was used to be presented of tannins on the extracts, Afew drops of ferric chloride reagent were added for 3 ml of extract. A blue black color refereed to the present of tannins.

**Alkaloids Test:** A few drops of Marqus reagent (prepared from mixing 0.5 ml of formaldehyde with 5ml of concentration H2SO4), added to the 5 ml of extract. Turbidity refereed to the present of alkaloids [14].

**Saponins Test:** 3 ml of extractwas added to the 2 ml of Ferric chloride, awhite residue to be formed as evidence to the present of Saponins [15].

**Flavonoids Test:** Flavonoids test were implement in conformity with [15]. 2 ml of extract mix with Alcoholic KOH (0.5 mol.), a yellow color as proofed to the present of Flavonoids.

**Glygosides Test:** 0.5g of dried extract was dissolved in 2ml of glacial acetic acid containing one drop of Ferric chloride solution and then under laid with 1 ml of concentration H2SO4A brown ring indicated the present of Glycosides [16]. All chemicals used (Purchased from Watin – Biolife Company produced by Jalil Medicals Company).

## RESULTS

This investigation of antimicrobial activity was performed on six different extracts of *M. sativa*as shown in (Table 1). The screening step in the preliminary study for antimicrobial activity was done using the agar well diffusion method. The inhibition activity was determined by measuring the diameter of clear zone. The diameter of the clear zone indicated the inhibition activity. The hot aqueous extract is the most effective against *S. aureus* followed by cold aqueous extract showed significant antibacterial activity against *B.* cereus, *Salmonella* typhimurium, *K. pneumoniae*, Ps.*aerogenes* and *E. coli*. Followed by ethanol extract followed by acetone extract followed by chloroform and ether extract while the ether extract did not show any significant antibacterial or antifungal activity against all examined microbes except *Enterococcus* as shown in Table (2). However Ethanol extract is the only extract showed high antifungal effect against *C. albicans*. Phytochemical screening indicated that the ethanolic extract most abundantly contained tannins, saponins and flavonoids, alkaloids and glycoside. as shown in Table (3).

## DISCUSSION

The use of herbs in medicine is common all over the world. About one third of adults in the world use alternative therapies, including plants which may be used singly or combined in mixtures. In the contrary to chemical drugs, plant sainted to be non-toxic, due to its natural origin. However, problems may originate due to adulteration, contamination, toxicity, substitution; misidentification and lack of standardization [17]. This undesirable fact encourage the study of medicinal plants and plant compounds used in medicine.

Table 1: List of *Medicago sativa* different extracts used to evaluate antimicrobial activity.

| No | Name | Colour of used extract |
|---|---|---|
| 1 | Ether | Light green |
| 2 | Ethanol | Dark green |
| 3 | Chloroform | Green yellow |
| 4 | Acetone | Green yellow |
| 5 | Hot aqueous extract | Green |
| 6 | Cold aqueous extract | Green |

Table 2: Antimicrobial activity of *Medicago sativa* different extracts against some animal pathogen in (mm).

| | | Type of extract | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Typeofmicroorganism | | ETH | A | C | E | CAE | HAE | P | Ci | N | D.W | DMSO |
| *G-Ve* | *E. coli* | 0 | 14 | 11 | 0 | 20 | 0 | 0 | 20 | 0 | 0 | 0 |
| | *Salmonella* | 0 | 7 | 11 | 0 | 25 | 21 | 15 | 30 | 0 | 0 | 0 |
| | *Enterococcus* | 14 | 11 | 0 | 13 | 0 | 32 | 20 | 35 | 0 | 0 | 0 |
| | *Ps.aerogens* | 17 | 14 | 0 | 0 | 12 | 19 | 0 | 30 | 0 | 0 | 0 |
| *G+Ve* | *Bacillus* | 20 | 11 | 0 | 0 | 28 | 30 | 0 | 34 | 0 | 0 | 0 |
| | *S aures* | 0 | 19 | 0 | 0 | 0 | 22 | 11 | 25 | 0 | 0 | 0 |
| | *S.pyogens* | 15 | 12 | 0 | 0 | 10 | 0 | 0 | 0 | 0 | 0 | 0 |
| | *K. pneumoniae* | 25 | 11 | 0 | 0 | 20 | 19 | 0 | 38 | 0 | 0 | 0 |
| *fungi* | *C. albicans* | 30 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 16 | 0 | 0 |

A= acetone/ C= chloroform/E= ether/ETH= ethanol/CAE= Cold aqueous extract/HAE= Hot aqueous extract/P= penicillin/Ci= Ciprofloxacin/N= Nystatin/DW= distelled water/ DMSO=dimethyle sulfoxide.

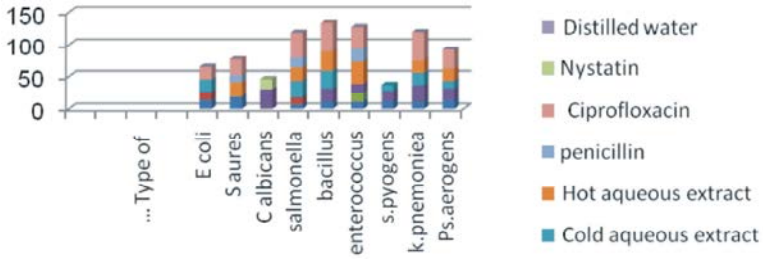


Fig. 1: Antimicrobial activity of *Medicago sativa* different extracts against some animal pathogen in (mm).

Table 3: The phytochemical compounds in ethanolic extract of *Medicago sativa*

| Plant extracts | Phytochemical tests*Medicagosativa* |
|---|---|
| Flavonoids Test | + |
| Alkaloids Test | + |
| Glygosides Test | + |
| Saponins Test | + |
| Tannins Test | + |

+ (contain this phytochemical compound)

However the revolution of synthetic antibiotics, the infections due to bacteria and fungi are still of major concern in medicine, due to the presence of antibiotic resistant strains which increasing the interest to use natural products due to their better biodegradability and availability [18].

In this regard, the study was performed to investigate the antimicrobial activity of *Medicago sativa* aqueous and solvent extracts on some medically important animal pathogen (*S. aureus, E. coli, S. pyogens, Pseudomonas aerogens, Salmonella typhimurium, Enterococcus, B. cereus, K. pneumoniae and C. albicans)* by agar well diffusion method.

All plant parts in order to perform their physiological activities form some chemicals by themselves, in the present study, *M. sativa* produce different kinds of secondary metabolites which play an important role medicinally.

A lot of studies involve extraction of the active component in the plants using organic solvents. But plants as used in traditionally, using organic solvent extraction for the antibacterial properties should not be done often; therefore, in the present study the commonly used plants extracts are made with hot and cold distilled water in addition to organic solvent extracts and tested for its antimicrobial effect against *S. aureus*, *E. coli and C. albicans.*

All plants used in the traditional medicine mostly have moderate antimicrobial activities as reported earlier [19-21].

According to Venkataswamy *et al*. [22] the Gram-positive bacterial strains were more susceptible to the activity of the aqueous *M. sativa* leaf extracts when compared to gram negative bacteria. This may be explained due to the fact that these two groups have different cell wall structure. The ability of tannin compounds which reported to be present in *M. sativa* disintegrates the bacterial colonies, by its interference with the bacterial cell wall.

Considering the above results, our result was found the maximum antibacterial activity in *Medicago sativa* against Gram positive *S.aureus* as comparing to Gram negative*E.coli*.as shown in Table (2) and Figure (1)

The antimicrobial activity of saponins isolated from *M. Sativa* against selected medically important yeasts, Gram-positive and -negative bacteria has been investigated. Increasing antibiotic activity was observed going from the saponin extracts. Activity was found especially high against Gram-positive bacteria (B.cereus) [8]

(Xiao-kang and Pinarosa ) mentioned that the better antibacterial activity of Alfalfa extract to *P. aeruginosa* and *S. dysgalactiae* where the antibacterial effects on *E.coli* and *S. aureus* were ordinary [23, 24].

However this investigation of antimicrobial activity was performed on six different extracts of *M. sativa* as shown in (Table 1). The screening step in the preliminary study for antimicrobial activity was done using the Agar well Diffusion Method. The diameter of the clear zone indicated the inhibition activity. The hot aqueous extract is the most effective against *S. aureus* followed by cold aqueous extract showed significant antibacterial activity against *B. cereus*, *Salmonella typhimurium*, *K. pneumoniae, Ps. aerogens* and *E. coli* followed by ethanol extract followed by acetone extract followed by chloroform and ether extract while the ether extract did not show any significant antibacterial or antifungal activity against all examined microbes except *Enterococcus* as shown in Table (2) and Figure (1).

The results of our study showed that ethanol extract of *Medicago sativa* possess very potential antifungal effect on *C. albicans* as shown in Table (2) and Figure (1).Also All phytochemical tests results indicated that *Medicago sativa* contain flavonoids, tannins, alkaloids, saponin and glycosides compounds as shown in Table (3)

Antimycobacterial properties of the plant could be due to the abundant flavonoids, saponins and tannins that were found in it Table (3). Phytochemical constituents such as tannins, saponins, flavonoids, alkaloids and several other aromatic compounds are secondary metabolites of plants that serve as defense mechanisms against predation by many microorganisms, insects and other herbivores [25].

Therefore, in order to obtain effective antimicrobial activity at low concentrations without any side effects on the health researches should focus on the optimization of purification and applications.

## CONCLUSIONS

So it could be concluded that the *Medicago sativa* extract exhibited remarkable antimicrobial activity against microbial pathogens and can be introduced as an alternative to chemical antimicrobial drugs, but it requires wider investigation. Also the data suggest that the ethanolic extract of *M. sativa* could be a rich source of antimicrobial agents, especially antifungals. Those results have been encourage to conduct additional tests to confirm the specific use of the studied extracts to treat the bacterial and fungal infections caused by bacteria and fungi found to be sensitive to our extracts.

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